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METHOD FOR PRODUCING SUBSTANCE UTILIZING MICROORGANISM

Background of the Invention

Field of the Invention

The present invention relates to a method for producing a substance utilizing a microorganism. In the present invention, the microorganism is typically selected from bacteria belonging to the genus *Escherichia* or coryneform bacteria, which are conventionally used for production of substances. The substance to be produced may be selected from those conventionally produced by using microorganisms, for example, L-amino acids, nucleic acids, antibiotics, vitamins, growth factors, physiologically active substances and so forth. The present invention discloses means for improving productivity of final target substances in methods for producing substances by utilizing microorganisms.

Description of the Related Art

Many organisms acquire energy required for the life activities by respiration. In the respiration of microorganisms, various enzyme complexes generally function according to their respective species or growth environment, and energy acquisition efficiency also varies significantly. Carbohydrates, proteins and aliphatic acids are made into acetyl-CoA by glycolysis, β-oxidation and so forth, and decomposed in the citric acid cycle. The energy preserved therein may be in the form of NADH. This may be used for proton excretion from

microbial cells with the aid of NADH dehydrogenase (NDH) and an electron transfer system consisting of oxidoreductases. A proton concentration gradient is, therefore, formed between the inside and the outside of the cytoplasmic membrane. This proton concentration gradient is used as a driving force of adenosine triphosphate (ATP) synthesis. At this time, a pathway showing high proton excretion ability and a pathway showing low proton excretion ability exist among the pathways of electron transfer depending on the combination of NDH and the oxidoreductases. It is considered that a pathway of high proton excretion ability shows high energy efficiency and a pathway of low proton excretion ability shows low energy efficiency. Thus, one kind of microorganism simultaneously contains a plurality of respiratory chain electron transfer pathways in parallel, and those pathways include those of high energy efficiency and low energy efficiency.

There are two kinds of NDHs and two kinds of terminal oxidases in the respiratory chain of *Escherichia* coli for an aerobic condition. That is, as for NDH, NDH-1 (encoded by *nuo* operon) of high energy efficiency and NDH-II (encoded by *ndh*) of low energy efficiency are known. Further, as for terminal oxidase, there is known cytochrome bo type oxidase (encoded by *cyoABCD* operon) which has been classified into SoxM type (Castresana, J. and Saraste, M., *Trends in Biochem. Sci., 20,* 443-448 (1995)). This oxidase shows high energy efficiency. Further, there exists cytochrome bd type oxidase (encoded by *cydAB)* which shows low energy efficiency. Although it is known that the expression amounts of these respiratory chain enzymes vary in response to growth environment (Minagawa et al., *The Journal of Biological Chemistry*, 265:11198-11203 (1990); Tseng et al., *Journal of* Bacteriology, 178:1094-1098 (1996); Green *et al., Molecular Microbiology,* 12:433-444 (1994); Bongaerts et al., *Molecular Microbiology,*

16:521-534 (1995)), there are still many unknown points about the physiological meaning of their expression patterns.

Further, in *Corynebacterium glutamicum*, there is a cytochrome bcl complex, and presence of at least two kinds of terminal oxidases, SoxM type oxidase and cytochrome bd type oxidase, is confirmed (The Second Symposium Concerning Metabolic Engineering, Lecture Abstracts, 1999). This shows that the electron transfer pathway form quinone pool to oxygen molecule include two kinds of pathways, a pathway utilizing cytochrome bcl complex and SoxM type oxidase and a pathway utilizing only the cytochrome bd type oxidase. It is considered that the former is an electron transfer pathway of high energy efficiency, in which proton transfer value for transfer of one electron is high, and the latter is an electron transfer pathway of low energy efficiency, in which proton transfer value for transfer of one electron is low.

As for the terminal oxidase of *E. coli*, if growth yields in an aerobic culture of a mutant strain having only the cytochrome bo type oxidase, mutant strain having only the cytochrome bd type oxidase and a wild strain having the both are compared, the growth yield will be the lowest in the mutant strain having only the cytochrome bd type oxidase, and it depends on the kind and energy acquisition efficiency of terminal oxidase (Annual Meeting of the Society for fermentation and Bioengineering Japan, 1995, Lecture Abstracts, No. 357).

Further, the energy efficiency of deficient mutants of some respiratory chain enzymes has been reported *(Calhoun et al., Journal of Bacteriology, 175:3020-3925 (1993)).*

However, there are no reports about the change of energy efficiency by amplification of a respiratory chain gene providing high efficiency such as those for NDH-I and SoxM type oxidase, and it has not been reported that there existed an attempt to utilize it for production of

substances. Furthermore, no attempts have been made to utilize deletion of a respiratory chain enzyme of low efficiency such as NDH-II and cytochrome bd type oxidase for production of substances.

Summary of the Invention

Energy is required for biosynthesis of substances such as L-amino acids and nucleic acids in living bodies. Most energy used consists of the reducing powers of NADH, NADPH and so forth and energy preserved as ATP. Therefore, the inventors of the present invention conceived that, if energy supply utilized in the production of target substances was increased in methods for producing target substances utilizing microorganisms, productivity of the target substances would be improved. Based on such a conception, an object of the present invention is to construct a microorganism showing improved energy efficiency and provide a method for producing a target substance by utilizing it.

The inventors of the present invention conceived that a microorganism showing increased energy supply could be constructed by enhancing a respiratory chain pathway showing high energy acquisition efficiency or making deficient a respiratory chain pathway showing low energy acquisition efficiency. Specifically, *E. coli*, strains considered to have improved energy efficiency were prepared by amplifying a gene coding for cytochrome bo type oxidase as a respiratory chain enzyme of high energy efficiency, or deleting a gene coding for NDH-II as axespiratory chain enzyme of low energy efficiency. Then, L-amino acid production was performed by using them and it was found that the L-amino acid productivity was improved in strains whose energy efficiency was improved. Thus, the present invention was accomplished.

That is, the present invention provides the followings.

(1) A method for producing a target substance utilizing a microorganism comprising culturing the microorganism in a medium to produce and accumulate the target substance in the medium and collecting the target substance, wherein the microorganism is constructed from a

parent strain of the microorganism having a respiratory chain pathway of high energy efficiency and a respiratory chain pathway of low energy efficiency as respiratory chain pathways, and the microorganism is a mutant strain or a genetic recombinant strain having either one or both of the following characteristics:

(A) The respiratory chain pathway of high energy efficiency is enhanced,

(B) The respiratory chain pathway of low energy efficiency is deficient.

(2) The method for producing a target substance according to (1), wherein the respiratory chain pathway of high energy efficiency is enhanced by increasing a copy number of a gene coding for an enzyme involved in the respiratory chain or modification of an expression regulatory sequence of the gene.

(3) The method for producing a target substance according to (1) or (2), wherein the respiratory chain pathway of low energy efficiency is made deficient by disruption of a gene coding for an enzyme involved in the respiratory chain.

(4) The method for producing a target substance according to any one of (1) to (3), wherein enzymes of the respiratory chain of high energy efficiency include SoxM type oxidase, bcl complex, NDH-1 or two or three kinds of them.

(5) The method for producing a target substance according to any one of (1) to (4), wherein enzymes of the respiratory chain of low energy efficiency include cytochrome bd type oxidase, NDH-II or both of them.

(6) The method for producing a target substance according to any one of (1) to (5), wherein activity of SoxM type oxidase is enhanced and NDH-II is made deficient in the microorganism.

(7) The method for producing a target substance according to any one of Claims (1) to (6), wherein the SoxM type oxidase is cytochrome bo type oxidase.

(8) The method for producing a target substance according to any one of (1) to (7), wherein the microorganism is a bacterium belonging to the genus *Escherichia* or coryneform bacterium.

(9) The method for producing a target substance according to any one of (1) to (8), wherein the target substance is an L-amino acid or nucleic acid.

According to the present invention, in a method for producing a target substance utilizing a microorganism comprising culturing the microorganism in a medium to produce and accumulate the target substance in the medium and collecting the target substance, productivity of the target substance can be improved based on a principle different from conventional strategy.

Brief explanation of the Drawings

Fig. 1 shows construction of plasmid pTS-Δndh for producing NDH-II gene disrupted strain.

Fig. 2 shows construction of pMAN997.

Detailed Description of the Invention

Hereafter, the present invention will be explained in detail.

The substance produced by the production method of the present invention is not particularly limited so long as it is a substance that can be produced by a microorganism.

Examples thereof include, for example, various L-amino acids such as L-threonine, L-lysine, L-glutamic acid, L-leucine, L-isoleucine, L-valine and L-phenylalanine; nucleic acids such as guanylic acid and inosinic acid; vitamins; antibiotics; growth factors; physiologically active substances and so forth.

The microorganism used for the present invention is a microorganism having such an ability to produce a target substance as described above, constructed from a parent strain of a microorganism having a respiratory chain pathway of high energy efficiency and a respiratory chain pathway of low energy efficiency as respiratory chain pathways, and having either one or both of the following characteristics:

(A) The respiratory chain pathway of high energy efficiency is enhanced,

(B) The respiratory chain pathway of low energy efficiency is deficient.

In general, microorganisms including *E. coli* and coryneform bacteria simultaneously contain a plurality of respiratory chain electron transfer pathways in parallel, and those pathways include those of high proton transfer value and those of low proton transfer value per electron. In *E. coli*, for example, as for an electron donor of NADH, there are NDHI and NDHII as NADH dehydrogenase that catalyzes the proton transfer from NADH to quinone pool. Among these, NDHI shows high energy efficiency, and NDHII shows low energy efficiency. That is, NDHII shows a molecular number of protons that can be excreted with one electron (proton transfer value) is 0, whereas that of NDHI is considered to be 2.

In the present invention, such a pathway showing a high proton transfer value per electron as described above, i.e., a respiratory chain pathway of high energy efficiency, is enhanced, and a respiratory chain pathway of low energy efficiency is made deficient. The

respiratory chain pathway of high energy efficiency can be enhanced by enhancing activity of a respiratory chain enzyme involved in the respiratory chain pathway. The respiratory chain pathway of low energy efficiency can be made deficient by reducing or eliminating activity of a respiratory chain enzyme involved in the respiratory chain pathway.

The respiratory chain enzyme involved in a respiratory chain pathway is not particularly limited so long as it is an enzyme constituting the respiratory chain pathway. Specifically, examples thereof include dehydrogenases that catalyze electron transfer from an electron donor to quinone pool such as ubiquinone, dimethylmenaquinone and menaquinone, and oxidases that catalyze electron transfer from a quinone pool to electron donor.

The oxidases that catalyze a reaction producing a water molecule by electron transfer from quinone pool are classified into SoxM type (bo type) and bd type. The proton transfer value of the bo type is 2, whereas that of the bd type is 1. Therefore, the bo type shows higher energy efficiency.

In the present invention, the terms "high" and "low" used for energy efficiency are not used with absolute meanings, but they are used to mean relative conceptions as described above.

Means for enhancing activity of a respiratory chain enzyme of high energy efficiency, and means for reducing or eliminating activity of a respiratory chain enzyme of low energy efficiency will be explained hereafter.

In order to enhance activity of a respiratory chain enzyme of high energy efficiency, for example, a recombinant DNA can be prepared by ligating a gene fragment coding for the enzyme with a vector functioning in a cell of microorganism, preferably a multi-copy type vector, and introduced into the microorganism to transform the cell. The copy number of the

gene coding for the enzyme in the cell of the transformant strain is thereby increased, and as a result, the enzymatic activity is amplified. This procedure will be explained hereafter by exemplifying cyo operon *(cyoABCDE)* coding for a cytochrome bo type oxidase as a gene of respiratory chain enzyme of high energy efficiency.

The sequence of cyo operon of *E. coli* was already reported (Chepuri *et al., The Journal of Biological Chemistry,* 265:11185-11192 (1990)); and therefore the operon can be cloned based on that sequence. It is also possible to use a gene of bacterium belonging to the genus *Escherichia,* or a gene derived from other organisms such as coryneform bacteria as the cyo operon.

As a vector used for the gene cloning and introduction of gene into microorganism, for example, a plasmid autonomously replicable in *E.* coli cells can be used. Specific examples thereof include pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pSTV29 and so forth. For the gene introduction into coryneform bacteria, a shuttle vector autonomously replicable in coryneform bacteria and *E. coli* can be preferably used. Examples of plasmids autonomously replicable in coryneform bacteria are listed below.

pAM 330 (cf. Japanese Patent Unexamined Publication (Kokai) No. 58-67699)

pHM 1519 (cf. Japanese Patent Unexamined Publication No. 58-77895)

pAJ 655 (cf. Japanese Patent Unexamined Publication No. 58-192900)

pAJ 611 (cf. Japanese Patent Unexamined Publication No. 58-192900)

pAJ 1844 (cf. Japanese Patent Unexamined Publication No. 58-192900)

pCG 1 (cf. Japanese Patent Unexamined Publication No. 57-134500)

pCG 2 (cf. Japanese Patent Unexamined Publication No. 58-35197.)

pCG 4 (cf. Japanese Patent Unexamined Publication No. 57-183799)

pCG11 (cf. Japanese Patent Unexamined Publication No. 57-183799)

pHK4 (cf. Japanese Patent Unexamined Publication No. 5-7491)

In order to ligate a DNA fragment containing the cyo operon and a vector to form a recombinant DNA, the vector is first digested with a restriction enzyme suitable for the ends of the cyo operon. The ligation is usually performed by using a ligase such as T4 DNA ligase.

To introduce the recombinant DNA prepared as described above into a microorganism, any known transformation methods can be employed. For instance, employable are a method of treating recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for *E. coli* K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)); and a method of preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for Bacillus subtilis (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene, 1, 153 (1977)). In addition to these, also employable is a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA followed by introducing the recombinant DNA into the cells, which method is known to be applicable to *Bacillus subtilis,* actinomycetes and yeasts (Chang, S. and Choen, S.N., *Molec. Gen. Genet., 168,* 111 (1979); Bibb, M.J., Ward, J.M. and Hopwood, O.A., *Nature, 274,* 398 (1978); Hinnen, A., Hicks, J.B. and Fink, G.R., *Proc. Natl. Sci., USA, 75,* 1929 (1978)). Transformation of coryneform bacteria can be attained by the electric pulse method (see Japanese Patent Unexamined Publication No. 2-207791).

The amplification of cytochrome bo type oxidase activity can also be attained by allowing existence of multiple copies of the cyo operon on chromosomal DNA of host. In order

to introduce multiple copies of the cyo operon into the chromosomal DNA of a microorganism such as bacteria belonging to the genus *Escherichia* and coryneform bacteria, homologous recombination is carried out by using a sequence whose multiple copies exist in the chromosomal DNA as targets. As sequences whose multiple copies exist in the chromosomal DNA, repetitive DNA, or inverted repeats existing at the end of a transposable element can be used. Also, as disclosed in Japanese Patent Unexamined Publication No. 2-109985, it is also possible to incorporate the cyo operon into transposon, and allow it to be transferred to introduce multiple copies of the cyo operon into the chromosomal DNA. By either method, the number of copies of the cyo operon within cells of the transformant strain increases, and as a result, cytochrome bo type oxidase activity is enhanced.

The enhancement of cytochrome bo type oxidase activity can also be attained by, besides being based on the aforementioned gene amplification, replacing an expression regulatory sequence of cyo operon such as a promoter with a stronger one (see Japanese Patent Unexamined Publication No. 1-215280). For example, *lac* promoter, *trp* promoter, *trc* promoter, *tac* promoter, P_R promoter and P_L promoter of lambda phage, *tet* promoter, *amyE* promoter and so forth are known as strong promoters. Substitution of these promoters enhances expression of the cyo operon, and hence the cytochrome bo type oxidase activity is enhanced. Enhancement of an expression regulatory sequence may be combined with increasing the copy number of the cyo operon.

The enhancement of activity of a respiratory chain enzyme of high energy efficiency can also be attained by introducing such a mutation that the intracellular activity of the enzyme

should be increased through a mutagenesis treatment of the microorganism. Examples of such a mutation include mutations of coding region increasing specific activity of enzyme, mutations in expression regulatory sequences increasing expression amount of gene and so forth. As the mutagenesis treatment, there can be mentioned methods utilizing treatment by ultraviolet irradiation or treatment with a mutagenesis agent usually used for mutation treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid.

In order to reduce or eliminate activity of a respiratory chain enzyme of low energy efficiency, a mutation is introduced into the gene of the enzyme so that the intracellular activity of the enzyme should be reduced or eliminated, or the gene on chromosome of microorganism is disrupted so that the gene should not function normally. Hereafter, by exemplifying *ndh* coding for NDH-II as a gene of a respiratory chain enzyme of low energy efficiency, the method of disrupting the *ndh* gene will be explained.

The sequence of *ndh* of E. coli was already reported (Young *et al., European Journal of Biochemistry,* 116:165-170 (1981)), and therefore the gene can be cloned based on the sequence. It is also possible to use a gene of a bacterium belonging to the genus *Escherichia,* or a gene derived from other organisms such as coryneform bacteria as the *ndh* gene.

A *ndh* gene on chromosome can be disrupted by transforming a microorganism with DNA containing a *ndh* gene modified with internal deletion so as not to produce NDH-II functioning normally (deletion type *ndh* gene), and allowing recombination between the deletion type *ndh* gene and the *ndh* gene on the chromosome. Such gene destruction by homologous recombination has already been established, and there are methods utilizing a linear DNA, a plasmid that contains a temperature sensitive replication control region and so forth. In the

present invention, the method utilizing a plasmid that contains a temperature sensitive replication control region is preferred.

A ndh gene on host chromosome can be replaced with the deletion type *ndh* gene as follows. That is, recombinant DNA is first prepared by inserting a temperature sensitive replication control-region, deletion type *ndh* gene and marker gene for resistance to a drug, with which recombinant DNA a microorganism is transformed. Further, the resultant transformant strain is cultured at a temperature at which the temperature sensitive replication control region does not function, and then the transformant strain can be cultured in a medium containing the drug to obtain a transformant strain in which the recombinant DNA is incorporated into the chromosomal DNA.

In such a strain in which recombinant DNA is incorporated into chromosomal DNA, the deletion type *ndh* gene is recombined with the *ndh* gene originally present on the chromosome, and the two fusion genes of the chromosomal *ndh* gene and the deletion type *ndh* gene are inserted into the chromosome so that the other portions of the recombinant DNA (vector segment, temperature sensitive replication control region and drug resistance marker) should be present between the two fusion genes. Therefore, the transformant expresses NDH-II, because the normal *ndh* gene is dominant in this state.

Then, in order to leave only the deletion type *ndh* gene on the chromosomal DNA, one copy of the *ndh* gene is eliminated together with the vector segment *(including* the temperature sensitive replication control region and the drug resistance marker) from the chromosomal DNA by recombination of the two *ndh* genes. In that case, the normal *ndh* gene is left on the chromosomal DNA, and the deletion type *ndh* gene is excised from the chromosomal DNA, or to

the contrary, the deletion type *ndh* gene is left on the chromosomal DNA, and the normal *ndh* gene is excised from the chromosome DNA. In the both cases, the excised DNA may be retained in the cell as a plasmid *when* the cell is cultured at a temperature at which the temperature sensitive replication control region can function. Subsequently, the cell is cultured at a temperature at which the temperature sensitive replication control region cannot function to drop out plasmid DNA, and *ndh* gene deletion mutant can be obtained.

Examples of the vector having a temperature sensitive replication origin for *E. coli* include, for example, the plasmid pMAN997 described in International Patent Publication W099/03988 and so forth, and examples of the vector having a temperature sensitive replication origin for coryneform bacteria include, for example, the plasmid pHSC4 disclosed in Japanese Patent Unexamined Publication No. 5-7491 and so forth. However, the plasmids are not limited to these, and other vectors can also be used.

Specific examples of such a microorganism as obtained in the manner described above include microorganisms of which SoxM type oxidase or NDH-1, or both of them are enhanced, microorganisms of which activity of cytochrome bd type oxidase or NDH-II, or activities of the both are reduced or eliminated, and microorganism of which SoxM type oxidase or NDH-1, or both of them are enhanced and activity of cytochrome bd type oxidase or NDH-II, or activities of the both are reduced or eliminated. More specifically, there can be mentioned, for example, *E. coli* of which activity of SoxM type oxidase is enhanced and NDH-II is made deficient. Examples of Soxm type oxidase include cytochrome bo type oxidase.

The microorganism used for the present invention is not particularly limited so long as it can be imparted with the aforementioned properties, and examples thereof include bacteria

belonging to the genus *Escherichia such* as *E. coli*, coryneform bacteria such as *Brevibacterium lactofermentum (Corynebacterium glutamicum), Bacillus* bacteria such as *Bacillus subtilis, Serratia* bacteria such as *Serratia marcescens*, yeast such as *Saccharomyces cerevisae* and so forth.

Specifically, there can be mentioned, when the fermentation product is L-threonine, *E. coli* VKPM B-3996 (RIA 1867) (refer to the U.S. Patent No. 5,175,107), *Corynebacterium acetoacidophilum* AJ12318 (FERM BP-1172) (refer to U.S. Patent No. 5,188,949) and so forth; for L-lysine, there can be mentioned *E. coli* AJ11442 (NRRL B-12185, FERM BP-1543) (refer to U.S. Patent No. 4,346,170), *E. coli* W3110 (*tyrA*) (this strain is obtained by eliminating plasmid pHATerm from *E. coli* W3110 (*tyrA*)/pHATerm (FERM BP-3653), refer to International Patent Publication W095/16042), *Brevibacterium lactofermentum* AJ12435 (FERM BP-2294) (U.S. Patent of No. 5,304,476), *Brevibacterium lactofermentum* AJ3990 (ATCC31269) (refer to U.S. Patent No. 4,066,501) etc.; for L-glutamic acid, there can be mentioned *E. coli* AJ12624 (FERM BP-3853) (refer to the French Patent Unexamined Publication No. 2,680,178), *Brevibacterium lactofermentum* AJ12821 (FERM BP-4172) (Japanese Patent Unexamined Publication No. 5-26811, French Patent Unexamined Publication No. 2,701,489), *Brevibacterium lactofermentum* AJ12475 (FERM BP-2922) (refer to U.S. Patent No. 5,272,067), *Brevibacterium lactofermentum* AJ13029 (FERM BP-5189) (refer to International Patent Application JP95/01586) etc.; for L-leucine, there can be mentioned *E. coli* AJ11478 (FERM P-5274) (refer to Japanese Patent Publication (Kokoku) No. 62-34397), *Brevibacterium lactofermentum* AJ3718 (FERM P-2516) (refer to U.S. Patent No. 3,970,519) etc.; for L-isoleucine, there can be mentioned *E. coli* KX141 (VKPM B-4781) (refer to the

European Patent Unexamined Publication No. 519,113), *Brevibacterium flavum* AJ12149 (FERM BP-759) (refer to U.S. Patent No. 4,656,135) etc.; for L-valine, there can be mentioned *E. coli* VL1970 (VKPM B-4411) (refer to European Patent Unexamined Publication No. 519,113), *Brevibacterium lactofermentum* AJ12341 (FERM BP-1763) (refer to U.S. Patent No. 5,188,948) etc.; for L-phenylalanine, there can be mentioned *E.* coli AJ12604 (FERM BP-3579) (Japanese Patent Unexamined Publication No. 5-236947, European Patent Unexamined Publication No. 488,424), Brevibacterium lactofermentum AJ12637 (FERM BP-4160) (refer to the French Patent Unexamined Publication No. 2,686,898) and so forth.

In the microorganism used for the present invention, depending on a target substance, activity of an enzyme involved in biosynthesis of the target substance may be enhanced. Further, activity of an enzyme disadvantageous for the production of the target substance may be reduced or eliminated.

A target substance can be produced by culturing such a microorganism as described above in a medium to produce and accumulate the target substance in the medium, and collecting the target substance.

The medium used for the production of target substance may be a conventionally used well-known medium selected depending on a microorganism to be utilized. That is, the medium may be a usual medium containing a carbon source, nitrogen source, inorganic ions, as well as other organic components, if necessary. Any special medium is not required for practicing the present invention.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose or starch hydrolysate; alcohols such as glycerol or sorbitol; organic acids such as

fumaric acid, citric acid or succinic acid and so forth.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; aqueous ammonia and so forth.

It is desirable to allow required substances such as vitamin B_1, L-homoserine and L-tyrosine or yeast extract to be contained in appropriate amounts as organic trace nutrients other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and so forth are added in small amounts, if necessary.

The culture can be performed under conventionally used well-known conditions selected according to a microorganism to be utilized. For example, the culture is preferably performed under an aerobic condition for 16-120 hours. The culture temperature is preferably controlled to be 25°C to 45°C, and pH is preferably controlled at 5-8 during the culture. Inorganic or organic, acidic or alkaline substances as well as ammonia gas or the like can be used for pH adjustment.

For collection of the metabolic product from the medium after the culture, any special methods are not required for the present invention. That is, the present invention can be practiced by using a combination of conventionally well-known ion exchange techniques, precipitation techniques and other techniques.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Cloning of cytochrrome bo type oxidase gene

The sequence of cyo operon *(cyoABCDE)* coding for the cytochrome bo type oxidase of E. coli had been already reported (Chepuri et al., *The Journal of Biological Chemistry, 265:*11185-11192 (1990)), and therefore the operon was cloned based on the sequence.

Specifically, the target *cyo* operon gene was obtained from the phage library of Kohara (Kohara et a1., Cell, 50:495-508 (1987)) containing the cyo operon. Phage DNA was obtained from the phage clone 147[2H5] of Kohara *containing* the operon using Wizard lambda prep (Promega). The obtained phage DNA 147[2H5] was digested with PshBI, and the obtained 5.5 kb fragment containing the *cyo* operon was blunt-ended, and inserted into the SmaI site of pMW119 (Nippon Gene) to clone the cyo operon containing a promoter region. In the obtained plasmid, the cyo operon was inserted in the reverse direction with respect to the lactose operon promoter on pMW119. This plasmid was designated as pMW(CYO)B.

The plasmid pMW(CYO)B was introduced into *E. coli* W3110 strain (obtained from the National Institute of Genetics, Mishima, Shizuoka, Japan) to obtain W3110/pMW(CYO)B. Ubiquinol oxidase activity present in the cell extracts of W3110 and W3110/pMW(CYO)B strains was measured as terminal oxidase activity by using a known method (Kita et al., The Journal of Biological Chemistry, 259:3368-3374 (1984)). The results are shown in Table l.

Table 1: Ubiquinol oxidase activity

Strain	Ubiquinol oxidase activity (mmol/min/m protein)
W3110/pMW119	0.28
W3110/pMW(CYO)B	0.56

It was found that the terminal oxidase activity was enhanced in the strain introduced with

pMW(CYO)B as shown in Table 1. This enhancement of the terminal oxidase activity is considered to be caused by the enhancement of cytochrome bo type oxidase activity through the enhancement of cyo operon.

Example 2: Acquisition of NDH-II deficient strain

In order to produce a NDH-II deficient strain, an internally cleaved partial sequence of NDH-II (disrupted type NDH-II gene) was prepared. The partial sequence of NDH-II was cloned based on the known sequence of the gene *ndh* coding for NDH-II of *E.* coli (Young et al., European Journal of Biochemistry, 116:165-170 (1981)).

Specifically, the disrupted type NDH-II gene was produced as follows (Fig. 1). First, a DNA fragment of about 2.4 kb containing the partial sequence of NDH-II was amplified from *E.* coli chromosomal DNA by PCR using ndh-1 (SEQ ID NO: 1) and ndh-2 (SEQ ID NO: 2) as primers. This fragment was cloned into pGEM-T vector.(Promega) to obtain pGEM-ndh. This pGEM-ndh was digested with restriction enzymes *EcoRI* and StuI, and the obtained DNA fragment of 0.5 kb was collected and ligated to pTWV229 (Takara Shuzo) digested with *EcoRI* and *SmaI* to obtain pTWV-ndh.

Then, pGEM-ndh was digested with a restriction enzyme *StuI*, and the obtained DNA fragment of 0.9 kb was collected and inserted into the *HincII* site of pTWV-ndh. Thus, there was obtained pTWVΔndh containing a part of the multi-cloning sites of pTWV229 in the partial sequence of *ndh.* The plasmid pTWVΔndh contained the *ndh* sequence inserted with a sequence of 17 by derived from pTWV229 at the *StuI* site in the *ndh* sequence. Subsequently, a fragment of 1.5 kb obtained by digesting pTWVΔndh with HindIII and *EcoRI* was inserted between the

HindIII and *EcoRI* sites of the temperature sensitive plasmid pMAN997 (refer to International Patent Publication WO 99/03988) to obtain pTS-Δndh. Homologous recombination was performed between this plasmid pTS-Δndh and the genome of W3110 strain as for *ndh* by a usual homologous recombination technique utilizing the temperature sensitivity of pTS-Δndh (Matuyama et al., *Journal of Bacteriology,* 162:1196 (1985)) to obtain a *W3110(ndh)* strain that did not express normal NDH-II protein because the sequence of 17 by derived from pTWV229 was inserted in the coding region of *ndh* on the genome. From *W3110(tyrA), tyrA* deficiency was introduced into the *W3110(ndh)* strain by P1 transduction using tetracycline resistance as a marker to obtain a *W3110(ndh,* tyrA) strain.

The aforementioned pMAN997 was obtained by exchanging the *VspI-HindIII* fragments of pMAN031 (J. *Bacteriol.,* 162, 1196 (1985)) and pUC19 (Takara Shuzo) (Fig. 2).

Further, while the W3110(tyrA) strain is detailed in European Patent Unexamined Publication No. 488424/1992, its preparation method will be briefly explained below.

The *E. coli* W3110 strain was obtained from the National Institute of Genetics (Mishima, Shizuoka). This strain was seeded on an LB plate containing streptomycin, and a strain that formed a colony was selected to obtain a streptomycin resistant strain. The selected streptomycin resistant strain and *E. coli* K-12 ME8424 strain were mixed, and cultured in a complete medium (L-Broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl) at 37°C for 15 minutes as stationary culture to induce conjugation. The *E. coli* K-12 ME8424 strain has the genetic traits of (HfrP045, thi, *relA1,* tyrA::Tn10, ung-1, *nadB),* and it can be obtained from the National Institute of Genetics. Thereafter, the culture was seeded in a complete medium (L-Broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar) containing streptomycin,

tetracycline and L-tyrosine, and a strain that formed a colony was- selected. This strain was designated as *E. coli* W3110(tyrA) strain European Patent Unexamined Publication No. 488424/1992 discloses many strains obtained by introducing a plasmid into in the above strain.

For example, a strain obtained by introducing a plasmid pHATerm was designated as E. coli W3110(tyrA)/pHATerm, deposited on November 16, 1991, at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305) (currently, the independent administrative corporation, the National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-5466) as an international deposition under the provisions of the Budapest Treaty, and received an accession number of FERM BP-3653. The E. coli W3110(tyrA) strain can be obtained by eliminating the plasmid pHATerm from the above strain in a conventional manner.

Example 3: Production of L-lysine

The plasmid pMW(CYO)B obtained in Example 1 was introduced into the W3110(tyrA) strain and the *W3110(ndh,* tyrA) strain obtained in Example 2 to obtain W3110(tyrA)/pMW(CYO)B and *W3110(ndh,* tyrA)/pMW(CYO)B, respectively. Similarly, pMW119 was introduced into W3110(tyrA) to obtain W3110(tyrA)/pMW119 strain. L-Lysine productivity of these W3110(tyrA)/pMW(CYO)B strain, W3110(ndh, tyrA)/pMW(CYO)B strain, and W3110(tyrA)/pMW119 as a control was evaluated by culture in flask. The culture was performed by using a medium having the following composition at 37°C for 24 to 48 hours

with shaking. The results are shown in Table 2.

(Medium composition)

Glucose	40 g/L
$MgSO_4 \cdot 7H_2O$	1 g/L
KH_2PO_4	1 g/L
$FeSO_4 \cdot 7H_2O$	0.01 g/L
$MnSO_4 \cdot 5H_2O$	0.01 g/L
Yeast Extract (Difco)	2 g/L
L-Tyrosine	0.1 g/L or 0.05 g/L

The medium was adjusted to pH 7.0 with KOH, and autoclaved at 115°C for 10 minutes. However, glucose and $MgSO_4 \cdot 7H_20$ were sterilized separately. Further, before the culture, 30 g/L of $CaCO_3$, according to Japanese Pharmacopoeia, which was subjected to dry sterilization at 180°C, and 100 μg/L of an antibiotics, ampicillin, were added to the medium.

Table 2: L-lysine production amount

Strain	L-Lys (g/L)
W3110(tyrA)/pMW119	0.29
W3110(tyrA)/pMW(CYO)B	0.48
W3110(ndh, tyrA)/pMW(CYO)	0.53

It was found that the L-lysine productivity was improved in the E. coli producing L-lysine by enhancing the cytochrome bo type oxidase activity. This is considered to be caused because energy acquisition efficiency was improved by enhancement of the respiratory chain

pathway of high energy efficiency, and the energy was used for the L-lysine production.

It was also found that the L-lysine productivity was improved in the *E.* coli producing L-lysine by making NDH-II deficient. This is considered to be caused because energy acquisition efficiency was improved by deficiency of the respiratory chain pathway of low energy-efficiency, and the energy was used for the L-lysine production.

Example 4: Production L-threonine

The plasmid pMW(CYO)B obtained by the aforementioned method was introduced into an L-threonine producing bacterium, *E.* coli VKPM B-3996 (RIA 1867) (refer to U.S. Patent No. 5,175,107, hereafter referred to as "B-3996" strain) to obtain B-3996/pMW(CYO)B strain. The B-3996 strain harbored a plasmid pVIC40 (International Patent Publication W090/04636) obtained by inserting the threonine operon into a wide host-range vector plasmid pAYC32 containing a streptomycin resistance marker (refer to Chistorerdov, A.Y., Tsygankov, Y.D., Plasmid, 1986, 16, 161-167). The B-3996 strain was deposited at USSR Antibiotics Research Institute (VNIIA) under a registration number of RIA1867.

As a control, B-3996/pMW119 was obtained by introducing pMW119 into B-3996. The L-threonine productivity of these B-3996/pMW(CYO)B and B-3996/pMW119 was evaluated by culture in flask. The culture was performed by using a medium having the composition mentioned in Table 3 at a temperature of 37°C for 38 hours with stirring at 114-116 rpm. Component A, Component B and Component C mentioned in Table 3 were prepared and sterilized separately, and then they were cooled and mixed in a ratio of 16/20 volume of Component A, 4/20 volume of Component B and 30 g/L of Component C. The results are

shown in Table 4.

Table 3: Threonine production medium

A	$(NH_4)_2SO_4$	16 g/L
	KH_2PO_4	1 g/L
	$FeSO_4 \cdot 7H_2O$	0.01 g/L
	$MnSO_4 \cdot 4H_2O$	0.01 G/L
	Yeast Extract (Difco)	2 g/L
	L-Isoleucine	50 mg/L
	Nicotinic acid	10 mg/L
	Adjusted to pH 7.0 with KOH and autoclaved at 115°C for 10 minute (16/20 volume)	
B	20% glucose autoclaved at 115°C for 10 minute (4/20 volume)	
	$MgSO_4 \cdot 7H_2O$	1 g/L
C	$CaCO_3$ according to Japanese Pharmacopoeia, subjected to dry sterilization at 180°C (30 g/L)	
	Antibiotics (100 μg/L of streptomycin and 5 μg/L of kanamycin)	

Table 4: Production amount of L-threonine

Strain	L-Thr (g/L)
B-3996/ MW119	13.1
[B-3996/pMW(CYO)B	14.3

It was found that the L-threonine productivity of L-threonine producing E. coli could be improved by enhancing cytochrome bo type oxidase activity.

Example 5: Production of L-phenylalanine

A plasmid pACMAB was collected from the E. coli W3110(tyrA)/pACMAB, pBR-aroG4 strain according to a usual purification method for plasmid. The plasmid was a plasmid obtained by inserting a DNA fragment containing a gene for desensitized type chorismate

mutase/prephenate dehydratase (CM-PDH) in the proper L-phenylalanine biosynthesis system between the BamHI and HindIII cleavage sites of the plasmid vector pACYC184 (Apr) (refer to International Patent Publication W097/08333). The W3110(tyrA)/pACMAB, pBR-aroG4 strain (designated as AJ12604) was deposited on January 28, 1991 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305) and received an accession number of FERM P-11975. Then, it was transferred to an international deposition under the provisions of the Budapest Treaty on September 26, 1991, and received an accession number of FERM BP-3579.

The plasmid pACMAB was blunt-ended by digestion with SalI. Into this, a blunt-ended DNA fragment containing the cyo operon of 5.5 kb, which was obtained from the aforementioned phage DNA 147[2H5] of Kohara by digestion with PshBI, was inserted. The obtained plasmid pACMAB-cyo was introduced into W3110(tyrA/pBR-aroG4. The obtained transformant strain was cultured in a medium for L-phenylalanine production (containing 20 g of glucose, 29.4 g of disodium hydrogenphosphate, 6 g of potassium dihydrogenphosphate, 1 g sodium chloride, 2 g of ammonium chloride, 10 g of sodium citrate, 0.4 g of sodium glutamate, 3 g of magnesium sulfate heptahydrate, 0.23 g of calcium chloride, 2 mg of thiamin hydrochloride, and 100 mg of L-tyrosine in 1 L of water, pH 7.0) at 37°C for 40 hours. L-Phenylalanine contained in the medium was quantified by high performance liquid chromatography. The results are shown in Table 5.

Table 5: Production amount of L-phenylalanine

Strain	L-P he (g/L)
W3110(tyrA)/pACMAB, BR-aroG4	3.9
W3110(tyrA)/pACMAB-cyo, pBR-aroG4	4.2

It was found that the L-phenylalanine productivity of the L-phenylalanine producing E. coli was improved by enhancing the cytochrome bo type oxidase activity.